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On the mechanism by which noradrenaline increases the activity of phosphofructokinase in isolated rat adipocytes

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We confirmed that, as reported by Sooranna & Saggerson [(1982) *Biochem. J.* **202**, 753–758], the affinity of 6-phosphofructo-1-kinase (PFK) for fructose 6-phosphate in an adipocyte extract was increased after incubation of the cells in the presence of noradrenaline. The participation of fructose 2,6-bisphosphate in this kinetic modification could be excluded, because the noradrenaline effect persisted after extensive gel filtration of the extracts and also because the treatment did not cause any change in the concentration of fructose 2,6-bisphosphate in the adipocytes. Oleic acid was found to be another potent positive effector of PFK in an adipocyte extract, with a K_a of 10 μM. Its effect was synergistic with that of fructose 2,6-bisphosphate and AMP, and was counteracted by serum albumin. Palmitic acid had a similar effect. We conclude that the large increase in fatty acid concentration caused by noradrenaline treatment is an explanation for the activation of phosphofructokinase at low fructose 6-phosphate concentrations in an adipocyte extract.

Sooranna & Saggerson (1982) have reported that the affinity of PFK for Fru6*P* was increased in extracts from adipocytes that had been exposed to noradrenaline. This effect was believed to be caused by an interaction of a ligand with PFK. Although this ligand could be removed with albumin, it was apparently not a fatty acid, since sodium palmitate did not mimic the effect of noradrenaline. It could also be Fru(2,6)P_2, a recently discovered potent positive effector of liver PFK (Van Schaftingen *et al.*, 1980*a*,*b*) which also acts on PFK species of numerous other origins (reviewed by Hers & Van Schaftingen, 1982).

A second change in the kinetic properties of PFK caused by noradrenaline and reported by Sooranna & Saggerson (1982) was a decrease in $V_{max.}$. This effect was not abolished by the presence of serum albumin, and appears therefore to operate through another mechanism than the activation at low substrate concentrations.

The purpose of the present work was to investigate the potential effect of noradrenaline to increase the concentration of Fru(2,6)P_2 in isolated adipocytes. Such an effect was not observed, but the stimulation of PFK in extracts of noradrenaline-treated adipocytes could be explained by a potent stimulation of the enzymic activity by fatty acids. A preliminary report of this work has been published (Lederer & Hers, 1983).

Abbreviations used: PFK, 6-phosphofructo-1-kinase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Fru6*P*, fructose 6-phosphate; Fru(1,6)P_2, fructose 1,6-bisphosphate; Fru(2,6)P_2, fructose 2,6-bisphosphate; Glc6*P*, glucose 6-phosphate.

Materials and methods

Chemicals

Fru6*P*, Glc6*P*, ATP, fatty acid-free serum albumin (fraction V), palmitic acid (sodium salt) and oleic acid were from Sigma (St. Louis, MO, U.S.A.). NADH, Hepes, auxiliary enzymes and collagenase were from Boehringer (Mannheim, Germany). 4-Methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside was from Koch–Light (Colnbrook, Bucks., U.K.). L-Noradrenaline bistartrate was from Winthrop Laboratories (Brussels, Belgium). Fru(2,6)P_2 was prepared by Dr. E. Van Schaftingen as described by Van Schaftingen & Hers (1981). A suspension of fatty acids that was stable for at least 3 h at 25°C was obtained by vigorous shaking in the presence of bovine serum albumin (10 mM-oleic acid in 50 μM-albumin; 10 mM-palmitate in 100 μM-albumin).

Preparation and incubation of adipocytes

The epididymal fat-pads from fed male Wistar rats weighing 140–150g were disaggregated with collagenase as described by Rodbell (1964), except that the glucose was omitted from the medium and that the concentration of serum albumin was 2%. The cells obtained from ten rats were pooled. An amount of adipocytes equivalent to one fat-pad was incubated for 30min with shaking in 5ml of Krebs–Henseleit (1932) bicarbonate buffer containing 5mM-glucose and fatty acid-poor albumin (20mg/ml). The gas phase was CO_2/O_2 (1:19). At the end of the incubation, the cells were isolated by centrifugation for 15s at 200*g* and immediately frozen in a solid-CO_2/acetone mixture.

Analytical procedure

The frozen cells were resuspended by vigorous shaking in 1.0ml of 50mM-Hepes/100mM-KF/15mM-EGTA (pH7.1). The suspension was centrifuged for 1min at 7000*g* in an Eppendorf centrifuge and the resulting extract (infranatant) was used for analytical purposes. PFK was immediately assayed by the decrease in A_{340}; 1ml of incubation mixture contained 50mM-Hepes, pH7.1, 100mM-KCl, 1mM-NH_4Cl, 0.16mM-NADH, 1.5mM-ATP, 6.5mM-$MgCl_2$, 1.25mM-AMP, 50μg of aldolase, 1μg of triosephosphate isomerase, 10μg of glycerol-3-phosphate dehydrogenase, 20μl of the adipocyte extract and Fru6*P* at the concentration indicated. Glc6*P* was also added at a concentration 3.5-fold that of Fru6*P*. Auxiliary enzymes were desalted by filtration through Sephadex G-25. The reaction was initiated by addition of ATP after 5min pre-incubation.

N-Acetyl-β-D-glucosaminidase was measured by the hydrolysis of methylumbelliferyl *N*-acetyl-β-D-glucosaminide under the conditions described by Leaback & Walker (1961). One unit of enzyme is the amount that converts 1μmol of substrate/min under the conditions of the assay. As a mean, 1ml of adipocyte extract contained 8.55m-units of *N*-acetylglucosaminidase. To correct for incomplete cell recovery or extraction, PFK activity is expressed as μmol of Fru(1,6)P_2 formed/min and per unit of *N*-acetylglucosaminidase (activity ratio). Fatty acids were measured by the method of Duncombe (1963) after extraction of 0.25ml of extract with 3ml of chloroform. [Fru(2,6)P_2] was measured as described by Van Schaftingen *et al.* (1982) in a mixture made of 100μl of extract in 100μl of 0.1M-NaOH and heated for 5min at 80°C.

Results

Data shown are illustrative of a series of experiments which gave consistently the same type of results. The typical changes in PFK kinetics caused by an incubation of the adipocytes in the presence of noradrenaline and previously des-

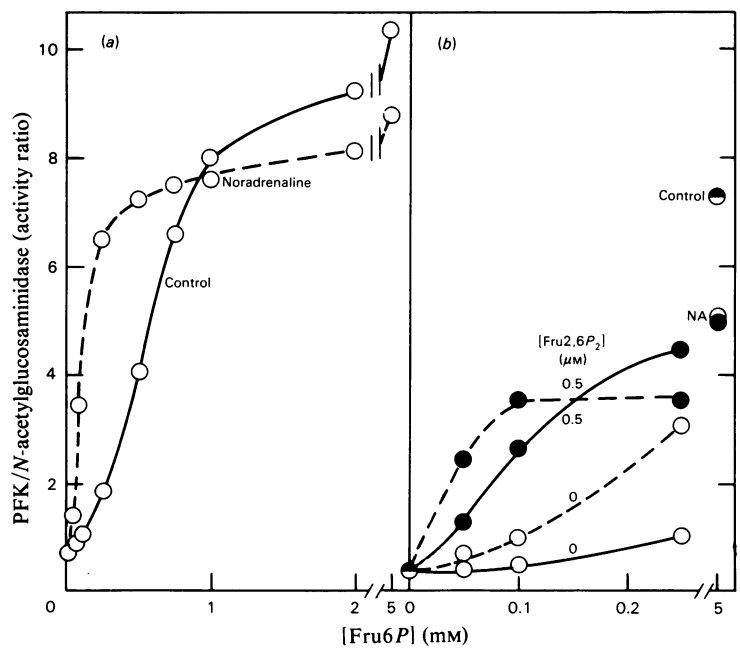

Fig. 1. *PFK activity in an adipocyte extract as a function of Fru6P concentrations*
The cells were incubated with (----) or without (——) noradrenaline. In (*b*), Fru(2,6)P_2 was added to the assay mixture at the concentration indicated. Abbreviation: NA, noradrenaline-treated.

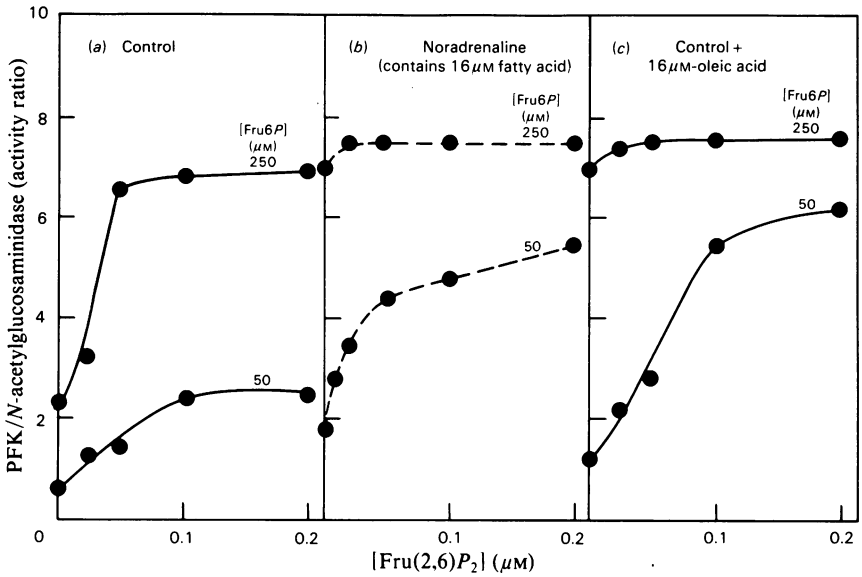

Fig. 2. *Effects of Fru(2,6)P_2 and of oleic acid on PFK activity in extracts of adipocytes incubated with* (----) *or without* (——) *noradrenaline*

PFK was measured at two concentrations of Fru6*P*. In (*c*), 16μM-oleic acid was added to the assay mixture. This was the concentration of fatty acids carried over with the extract in (*b*).

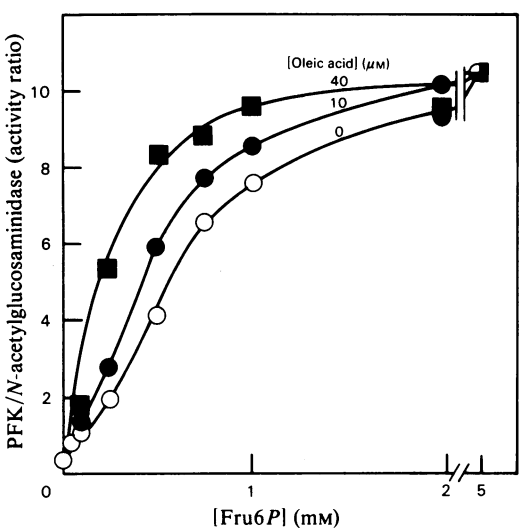

Fig. 3. *Effect of oleic acid on the affinity of PFK for Fru6P in an extract of control adipocytes*

cribed by Sooranna & Saggerson (1982) are illustrated in Figs. 1(*a*) and 1(*b*). In the experiments shown in Fig. 1(*a*), the K_m for Fru6*P* was decreased about 5-fold and $V_{max.}$ was decreased by about 10%. In the experiments shown in Fig. 1(*b*), the decrease in $V_{max.}$ was about 30%.

The effect of Fru(2,6)P_2 on the activity of PFK present in an adipocyte extract was investigated both in control and in noradrenaline-treated preparations (Figs. 1*b*, 2*a* and 2*b*). In both cases, the effect was to decrease K_m for Fru6*P* greatly, with no change in $V_{max.}$. It appears, however, that the PFK present in a noradrenaline-treated preparation was more sensitive to stimulation by Fru(2,6)P_2 than was the control (cf. Figs. 2*a* and 2*b*). In the treated preparation, the effect of Fru(2,6)P_2 was only observed at low concentrations of Fru6*P*, because at higher substrate concentrations the enzyme was already fully active in the absence of the stimulator (Fig. 2*b*). This indicates that the treatment caused the formation of a positive effector that acted synergistically with Fru(2,6)P_2 on PFK.

Experiments were then performed to check if this effector was Fru(2,6)P_2 itself. The following observations argued against this hypothesis. Firstly, gel filtration of the extracts did not remove the effect of noradrenaline treatment (Table 1). Secondly, the concentration of Fru(2,6)P_2 was measured in extracts from control and noradrenaline-treated adipocytes and found to be the same (approx. 0.2μM; results not shown). From the data of Fig. 2, one can calculate that a concentration of Fru(2,6)P_2 as high as 2.5μM in the extract (i.e. 0.05μM in the assay) would be required to account for the noradrenaline effect. Thirdly, it has also been verified that the amount of Fru(2,6)P_2

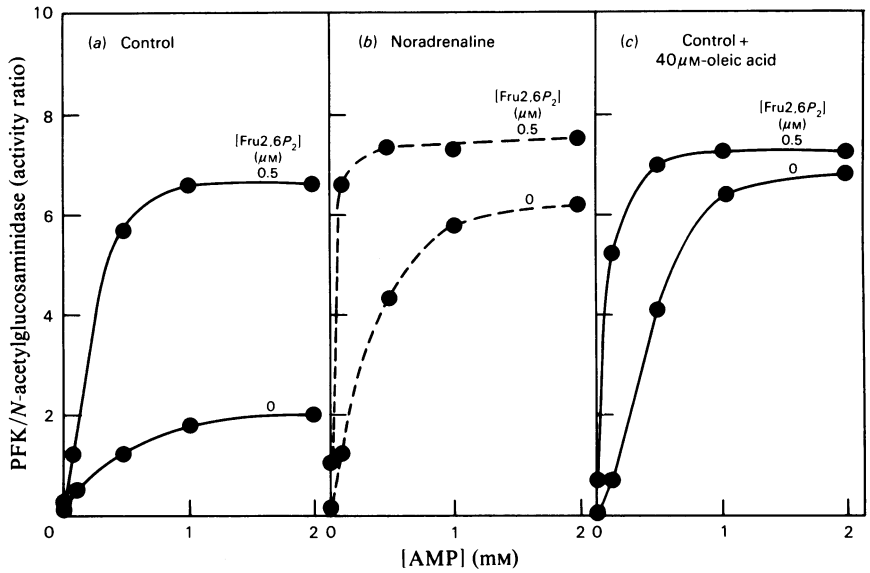

Fig. 4. *Effects of AMP, oleic acid and Fru(2,6)P_2 on PFK activity in extracts of adipocytes previously incubated with* (----) *or without* (——) *noradrenaline*

The concentration of Fru6*P* was 0.25mM.

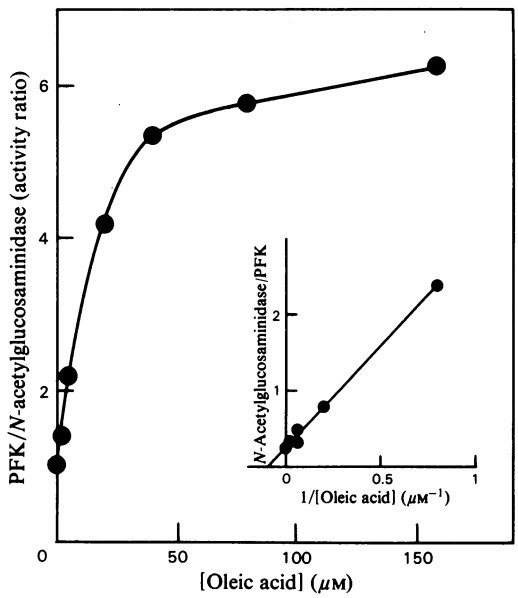

Fig. 5. *Effect of oleic acid on the activity of PFK measured at 0.25mM-Fru6P in an extract of control adipocytes*

formed from Fru6*P* and ATP during the assay of PFK did not exceed 2–3pmol/ml (result not shown).

Because of the negative results obtained with Fru(2,6)P_2, we decided to investigate the effect of fatty acids, the concentration of which is known to be greatly increased in adipocytes treated with noradrenaline. The effect of oleic acid (Fig. 3) and also of palmitate (results not shown) was to decrease the K_m for Fru6*P*, with no change in $V_{max.}$. This effect was synergistic with that of Fru(2,6)P_2 (cf. Figs. 2*a* and 2*c*) and with that of AMP (Fig. 4*c*). Fig. 4 also indicates that, in the absence of AMP, 0.5μM-Fru(2,6)P_2 had no effect on the activity of PFK in the control extract, but that 40μM oleic acid had a slight effect. When the assay was performed under our usual conditions (Fig. 5), a half-maximal effect was obtained at 10μM-oleic acid. This was well below the range of concentration of fatty acids that could be reached in the assay of PFK performed with extracts from noradrenaline-treated adipocytes (approx. 16μM, as shown in Table 1, and considering the 50-fold dilution of the extracts in the assay mixture). It is also apparent from Figs. 2 and 4 that the addition of oleic acid to a control extract mimicked the effect of the noradrenaline treatment.

The property of serum albumin in abolishing the noradrenaline effect is illustrated in Fig. 6. The same Figure shows that, when palmitic acid was added to that preparation, it had no stimulatory effect, indicating that PFK was already saturated with fatty acids. Palmitic acid could, however,

Table 1. *PFK activity and concentration of fatty acids in extracts of control and noradrenaline-treated adipocytes*
For gel filtration, 1 ml of extract was passed through a column (1.5 cm × 18 cm) of Sephadex G-25. Fractions (0.5 ml) were collected and the measurements were performed on the fractions that contained the highest activity of *N*-acetylglucosaminidase. The values were corrected for the dilution caused by gel filtration, as indicated by the *N*-acetylglucosaminidase activity. PFK values shown are the ratios of the activities measured at 0.25 mM- and 5 mM-Fru6*P*. Fatty acid concentrations lower than 100 μM could not be measured by the method of Duncombe (1963), and are indicated by n.d.

	Control		Noradrenaline	
	Before gel filtration	After gel filtration	Before gel filtration	After gel filtration
PFK ($v_{0.25}/V_{max.}$)	0.18	0.22	0.68	0.65
[Fatty acids] (nmol/ml)	n.d.	n.d.	800	750

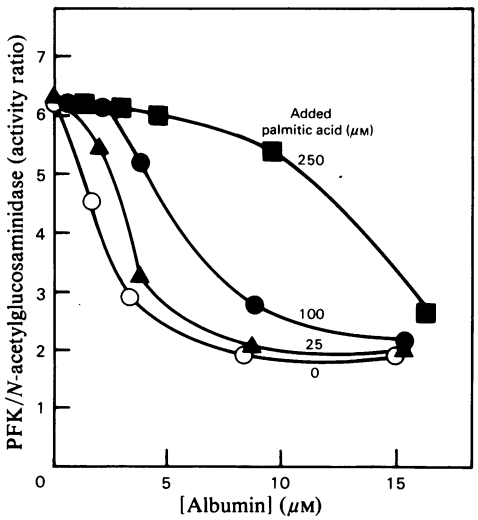

Fig. 6. *Effect of albumin on the activity of PFK measured in an extract of adipocytes previously treated with noradrenaline*
The enzyme was measured in the presence of added palmitic acid as indicated. The assay mixture also contained 18 μM endogenous fatty acids. The concentration of Fru6*P* was 0.25 mM.

counteract the inhibitory action of albumin. In the presence of 100 μM-palmitic acid, albumin became inhibitory only above 2.5 μM.

Discussion

Sooranna & Saggerson (1982) have previously concluded that the effect of noradrenaline to increase PFK activity is due to an interaction of a ligand. Since, as shown in the present work, the effect was not abolished by extensive gel filtration, the ligand cannot be Fru(2,6)P_2 or another low-molecular-weight and freely soluble effector of PFK. Furthermore, the concentration of Fru(2,6)P_2 was not affected by noradrenaline treatment. The ligand must therefore be of high molecular weight or be bound to a high-molecular-weight structure. Fatty acids fulfil the latter requirement, and several arguments indicate that they are the intermediate of the noradrenaline action. Firstly, they stimulated PFK activity in a crude adipocyte homogenate. Secondly, this action was synergistic with that of AMP and Fru(2,6)P_2, as was also that of the ligand present in the homogenate of treated adipocytes. Thirdly, their concentration was largely increased by the treatment. Fourthly, their effect was, like that of the endogenous ligand, abolished by serum albumin; for this reason, a demonstration of the positive effect of fatty acids on PFK activity requires that their solutions contain only a minimal amount of albumin. This condition was fulfilled in the present work, but apparently not in that of Sooranna & Saggerson (1982), who did not observe a stimulation of PFK by 1 mM-palmitate.

The mechanism by which fatty acids stimulate the activity of PFK in a crude extract of adipocytes is still obscure. Preliminary experiments performed in our laboratory have failed to demonstrate an effect of fatty acids on PFK purified from adipocytes. Surprisingly, the purified enzyme had a much lower K_m for Fru6*P* (0.1 mM) than did the crude enzyme. This indicates that the crude preparation might contain a protein that interacts with PFK and may be required for the fatty acid effect.

Whereas our work provides an explanation for the effect of noradrenaline in increasing the activity of PFK measured at low Fru6*P* concentrations, it does not explain the decrease in $V_{max.}$. A possible explanation for this second effect may be found in the report of Ramadoss *et al.* (1976) that fatty acids can cause a time-dependent inactivation of purified muscle PFK.

From the physiological point of view, our conclusion that an increase in fatty acid concentration explains the effect of noradrenaline in increasing the activity of PFK in the homogenate is in agreement with the observation that the incubation of adipose tissue in the presence of oleate or adren-

aline increases the glycolytic rate at the level of PFK (Halperin & Denton, 1969; Saggerson & Greenbaum, 1970). The advantage of this effect is presumably to favour the formation of glycerol 3-phosphate and the re-esterification of fatty acids when the concentration of the latter is increased.

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